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TITLE: USE OF LIPOSOMES FOR DIRECTED DRUG DELIVERY AGAINST ENTAMOEBA HISTOLYTICA

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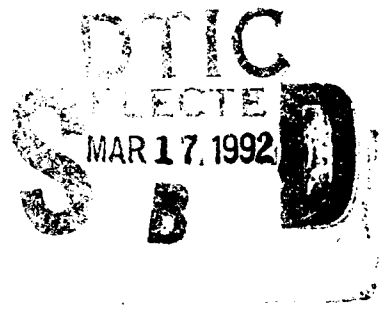

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The overall goal of this research has been to determine the feasibility and develop conditions for use of recognition specific liposomes as a means for targeted drug delivery against the intestinal parasite, *Entamoeba histolytica*. The specific research objectives were to: identify lipid molecules of mammalian target cell membranes that stimulate phagocytosis by *E. histolytica*; construct chemically defined liposomes that are selectively phagocytized by the parasite; determine the ability of recognition specific liposomes to deliver drugs and kill *E. histolytica* trophozoites in culture and protect cultured mammalian cells from destruction by the parasite; develop an animal model and use this to test the ability of drug loaded liposomes to eliminate the parasite *in situ*. Significant progress has been made on all but the last of these objectives. Two manuscripts of this work have been published and a third is in preparation. The result of this research is summarized below.

The ability of purified glycosphingolipids to enhance liposome stimulated *Entamoeba histolytica* actin polymerization was assessed as a means to define the specificity of mammalian cell membrane lipid glycan recognition by this parasite. Synthetic liposomes containing a variety of individual glycosphingolipids bearing neutral, straight chain oligomeric glycans with galactose or

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N-acetylgalactosamine termini stimulated rapid (90 sec) polymerization of amoeba actin. Glycans with terminal N-acetylglucosamine residues were not, or only weakly, stimulatory. Glycans with glucose, N-acetylglucosamine, galactose and N-acetylgalactosamine as the penultimate residue were recognized. Attachment of N-acetylneuraminate to the terminal residue of a stimulatory glycosphingolipid eliminated activity; attachment of fucose to the penultimate sugar reduced activity. Glycans with a terminal β1-4 or 1-3 glycosidic bond were most effective; glycans with terminal a1-4 or 1-3 glycosides were less effective. The activity of glycans with both β and a-linked terminal glycosides was inhibited by lactose, suggesting recognition of both configurations by a single amoeba protein. The ability of liposomes to stimulate actin polymerization reflected the extent of liposome phagocytosis.

Latex beads and liposomes carrying glycoproteins with carbohydrate sequences recognized by an *E. histolytica* galactose specific binding protein were assessed for their ability to adhere to trophozoites and to stimulate amoeba actin polymerization. Glycoprotein conjugated beads bound significantly to amoebae, but did not stimulate actin polymerization. Glycoprotein bearing liposomes bound and did enhance actin polymerization, as do recognized glycosphingolipid bearing liposomes. Liposome stimulated actin polymerization occurred only if the vesicle contained negatively charged phospholipid. It was concluded that both glycoprotein and glycosphingolipid glycans on the target cell surface are involved in attachment to *E. histolytica*, but do not themselves induce the transmembrane signals that lead to cytoskeleton activation and target destruction. This requires interaction with lipids of the target membrane bilayer.

Metronidazole loaded liposomes were tested for their ability to kill *E. histolytica* trophozoites and to protect cultured Chinese hamster ovary cells from destruction by the parasite. Metronidazole was soluble to a maximum of 4 mg/ml and liposome loading efficiency was 2.5%. Equivalent free solutions of drug were calculated from this information. Liposome delivered drug had the same efficacy as equivalent free drug solutions. However, it was determined that free metronidazole was lost from liposomes rapidly. Metronidazole-phosphate, a highly soluble derivative, was obtained and preliminary experiments indicate that it is retained within the lipid vesicles. It is currently being tested in continuing studies for its ability to kill *E. histolytica* and protect cultured cells.

Attempts were made to develop a rodent model that could be used to test the effectiveness of liposome delivered antiparasitic agents. Axenically cultivated and monoxenically (*E. coli*) cultivated *E. histolytica* trophozoites were inoculated into rat, hamster and gerbil cecum and into gerbil cecum following neomycin administration. None of these models resulted in large bowel colonization or invasion by *Entamoeba*.

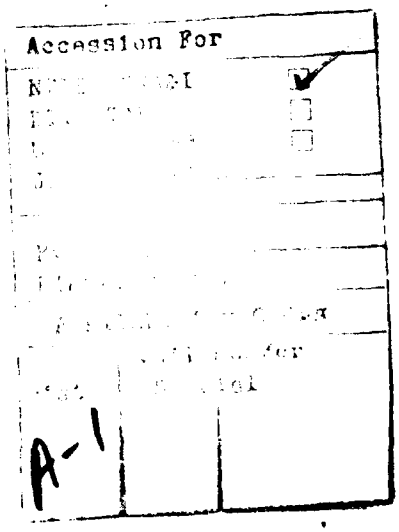

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TABLE OF CONTENTS

Scope and organization of this report.

This report is divided into three sections. The first section relates primarily to the first two research objectives, the construction and analysis of recognition specific liposomes, and describes work that has been published. The second section deals with the third research objective, tests of the ability of recognition specific, drug loaded liposomes, to selectively destroy *Entamoeba histolytica*, and describes studies that are still ongoing. This work will be continued with other resources and published. The third section describes work on the fourth objective, efforts to develop and animal model for intestinal amebiasis.

Section 1.

This section describes work on the identification of target cell membrane molecules that stimulate *Entamoeba histolytica* phagocytosis, the construction of recognition specific liposomes, and the roles of carbohydrate and phospholipids in stimulating *E. histolytica* phagocytosis of liposomes.

Introduction

Interaction of *Entamoeba histolytica* with mammalian cells is believed to be initiated by binding of amoeba membrane proteins to target cell surface carbohydrates (7, 13). The primary evidence for this is the inhibition by sugars of amoeba attachment and destruction of model target cells *in vitro*. The most effective monosaccharides are galactose (Gal) and N-acetylgalactosamine (GalNAc) (13). The most effective disaccharides are lactose (Lac) (3) and N-acetyllactosamine (LacNAc) (9). N-Acetylglucosamine (GlcNAc) and its oligomers, and melibiose (Galα1-6Glc), have also been reported to inhibit by some authors (3, 8, 11, 15) and not to by others (9).

Chinese hamster ovary (CHO) cell mutants with defined alterations of surface glycan sequences have also been used to explore *E. histolytica* carbohydrate recognition specificity. Li, et al. (9) employed a panel of lectin resistant mutants with altered Asn-linked carbohydrate chains to study target cell adherence to *E. histolytica*. Wild-type CHO cells contain the terminal sequence, NeuAcα2-3Galβ1-4GlcNAcβ1-3Man-. Mutants lacking the terminal NeuAc (increased Galβ1-4 termini) adhered more effectively to

E. histolytica trophozoites at 4°C than did wild-type cells or mutants lacking the terminal NeuAc-Gal (increased GlcNAc termini) or more of the terminal sequence (9). Ravdin et al. (14) assessed adherence and cytolysis of a panel of CHO cell mutants with alterations of both Asn-linked and Ser/Thr-linked glycans. They found that adherence and cytolysis was greatest with mutants bearing increased Galβ1-4 termini. On the basis of these findings and sugar inhibition studies, these groups concluded that N-acetyllactosamine units (Galβ1-4GlcNAc) were recognized most specifically by *E. histolytica*, a possibility that had been suggested earlier by Cano-Mancera and Lopez-Revilla (3).

To date, studies have focused on *E. histolytica* recognition of protein linked glycoconjugates. We demonstrated that liposomes prepared from human red blood cell (RBC) membrane lipids stimulate the same rapid, contact dependent polymerization of *E. histolytica* actin and phagocytosis that is triggered by contact with whole target cells (1, 2). The response to membrane liposomes was inhibited by the same sugars that block amoeba-whole cell interactions, suggesting that it was mediated by recognition of membrane glycosphingolipid glycans expressed on the surface of the vesicles (2). Here we describe the ability of synthetic liposomes formulated with a panel of individual membrane glycosphingolipids of known glycan sequence to stimulate amoeba actin polymerization. The intensity of the parasite's response was strongly affected by the structure of the glycosphingolipid glycan presented. The results demonstrate that *E. histolytica* interacts with mammalian cell membrane glycosphingolipids in liposomes and define glycosphingolipid glycan recognition specificity.

There is considerable evidence that an initial event in the destructive interaction of *E. histolytica* with mammalian cells *in vitro* is binding of an amoeba membrane lectin to terminal galactose (Gal) or N-acetylgalactosamine (GalNAc) residues of oligosaccharides on the surface of the target cell (13). Both glycoprotein (4, 9, 10, 14) and glycosphingolipid glycans (our observations) are recognized. The earliest biochemical response that has been detected following contact with the target cell is polymerization of amoeba actin (2); this is associated with extension of phagocytic membranes. Action of the amoeba cytoskeleton is known from cytochalasin inhibition studies to be required for effective parasite attachment and destruction of target cells (2, 11, 13). Therefore, contact induced actin

polymerization may be an essential event of the target cell destructive process.

E. histolytica actin polymerization is also stimulated by contact with liposomes prepared from target cell membrane lipids and by negatively charged synthetic liposomes (1). The following questions concerning the roles of carbohydrate ligands and of membrane lipids in eliciting the cytoskeleton response have not been thoroughly answered. Will binding of recognized glycans, only, if presented as a multivalent target of suitable size, trigger actin polymerization? Does the parasite distinguish between glycoprotein and glycolipid glycans on the target membrane surface in the mechanism of this response? We have addressed these questions using target models bearing glycans recognized by the amoeba lectin and without or with a lipid membrane.

Body

Experimental Methods

The following abbreviations are used: PBSS, 15 mM potassium phosphate-150 mM NaCl-5 mM $MgCl_2$-2 mM $CaCl_2$, pH 6.3; Gal, galactose; Glc, glucose; Man, mannose; Lac, lactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminate; LacNAc, N-acetyllactosamine.

Commercial lipids and sugars were obtained from Sigma Chemical Co., St. Louis, MO. Lipids were identified and checked for purity by TLC as described previously (1). Rhodamine-phalloidin was purchased from Molecular Probes, Eugene, OR.

Amoebae. *E. histolytica*, strain HM1-IMSS, was cultured axenically in TYI-S-33 medium (5) as described (2).

Glycosphingolipids. Glucosylceramide, galactosylceramide, lactosylceramide trihexosylceramide (CTH), (GlcNAc)trihexosylceramide ((GlcNAc)CTH), globoside, paragloboside (PG), Forssman, norhexaosylceramide (NHC), GM_3, and sialylparagloboside (SPG) were all prepared from human erythrocyte stroma as follows. Erythrocytes were lysed in hypotonic 0.1% acetic acid on ice and centrifuged. The washed pellet was homogenized in isopropanol:hexane:water (I:H:W) 55:25:20 v/v, and filtered. The organic extract filtrate was evaporated to dryness and brought up in a large volume of

chloroform:methanol (C:M), 2:1, whereupon 1/6 volume of deionized water was added and the suspension inverted and mixed well. After the phases separated, the upper phase was drawn off, evaporated to dryness, transferred in water to Spectrapore dialysis tubing (MW cutoff 2500) and dialyzed against water. The lower phase was also evaporated to dryness and brought up in a small volume of C:M. After dialysis of the upper phase for two days, this fraction was lyophilyzed and brought up in chloroform:methanol:water (C:M:W), 30:60:8.

Lower phase glycolipid preparation/HPLC chromatography: The lower phase was applied to an Iatrobeads column (porous silica gel, 10 μm diameter, Iatron, Tokyo), on a Varian HPLC and eluted with a gradient of I:H:W (55:40:5 to 55:25:20) at 2ml/min over 4 hr. In this manner, CMH, CDH, CTH and globoside were isolated in pure quantities. An impure Forssman fraction was subsequently purified by additional HPLC runs.

Upper phase. A DEAE Sephadex column (A-25, Sigma) was prepared after equilibrating the Sephadex in C:M:0.8 M sodium acetate, 30:60:8, overnight and washing in C:M:W, 30:60:8. The upper phase, dissolved in C:M:W, 30:60:8, was applied to the column and washed extensively with this solvent, then with MeOH. The monosialyl gangliosides were eluted with 0.05 M ammonium acetate in MeOH. This fraction was evaporated to dryness, transferred to a dialysis bag in water and dialysed 3 days. The dialyzed fraction was lyophilized and brought up in a small volume of C:M, 2:1.

HPLC of the monosialyl fraction. The fraction was chromatographed on an Iatrobeads column as above, and GM_3, SPG, and sialylnorhexaosylceramide (SNHC) were isolated in pure quantities. The SPG and SNHC were cleaved to PG and NHC respectively by heating in aqueous 1% acetic acid at 100^{o}C for 1 hr. (GlcNAc)CTH and agalactosyl-NHC were prepared by enzymatic cleavage of PG and NHC using jackbean β-galactosidase (Sigma) in 0.2 M citrate, pH 4.0, overnight at 37^{o}C.

Le^{x} pentasaccharide and dimeric Le^{x} were prepared from human colonic adenocarcinoma upper neutral fraction by HPLC in a manner similar to that above. Rabbit afucosyl B antigen was prepared from rabbit erythrocytes in the same way.

Preparation of Liposomes. RBC membrane liposomes were prepared by sonication as previously described (1). The total

lipid concentration of RBC membrane liposome suspensions was the same as that of synthetic liposomes based on total phosphorous content (6). Synthetic liposome suspensions contained 2.5 mM cholesterol, 2 mM sphingomyelin, 1.3 mM phosphatidylethanolamine, 1.2 mM dipalmityl phosphatidylcholine, 0.6 mM phosphatidylserine and 0.33 mM glycosphingolipid. Lipids, first dissolved and mixed in C:M:W (50:25:1), were dried at 45°C and sonicated in PBSS as described previously (1). Liposome suspensions were used within one week of preparation.

Assay of liposome stimulated amoeba actin polymerization. A simplification of the method described earlier (1) was used. Amoebae were washed and resuspended in PBSS at a concentration of 10^6 cells/ml. One hundred μl was placed in wells of a conical bottom 96-well plate, incubated at 25°C for 5 min, then challenged with 10 μl/well of liposome suspension. The amoebae were resuspended at 30 sec intervals during a 90 sec challenge, then fixed with an equal volume of 0.1% glutaraldehyde-7% formaldehyde in PBS. The cells were washed 5 times with PBS-0.1% Triton X-100 and stained with 0.17 μM rhodamine-phalloidin (1). Stimulation of *E. histolytica* actin polymerization was determined by counting the fraction of stimulated cells as previously described (1). RBC membrane liposomes and glycosphingolipid-free synthetic liposomes were included in each assay for comparisons. Relative stimulation was the fraction of cells stimulated by the test liposomes divide by the fraction stimulated by RBC membrane liposomes in the same assay. The values shown in the Figures are means ± SEM for the number of assays listed in Table 1. Statistical significance was determined using the student's t-test.

Liposome phagocytosis. Liposome phagocytosis was measured with carboxyfluorescein loaded liposomes as previously described (1).

Covalent conjugation of proteins to latex beads. Latex beads (Polysciences, Inc., Warrington, PA) the mean diameter of human erythrocytes were covalently conjugated with fetuin (N-acetylneuraminate (NeuAc)-Galβ1-4N-acetylglucosamine (GlcNAc) termini) and asialofetuin (Galβ1-4GlcNAc termini) at a ratio of 80 μg protein/10^8 beads, using dimethylcarbodiimide (Pierce, Rockford, IL) as described by the vendor. Agalactosyl-asialofetuin conjugated beads (GlcNAc termini) were prepared by treatment of the asialofetuin beads with jack bean β-galactosidase (Sigma Chemical Co., St. Louis, MO; 0.1 unit

TABLE 1. Glycosphingolipids tested.

Label	Name and formula (number of times assayed)
pl.	Glycosphingolipid-free liposomes (56)
rbc.	RBC membrane liposomes (56)
A.	Glucosylceramide (6) Glcβ1-ceramide
B.	Galactosylceramide (6) Galβ1-ceramide
C.	Lactosylceramide (19) Galβ1-4Glcβ1-ceramide
D.	Trihexaosylceramide (19) Galα1-4Galβ1-4Glcβ1-ceramide
E.	(GlcNAc)trihexaosylceramide (7) GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
F.	Globoside (19) GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-ceramide
G.	Paragloboside (40) Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
H.	Rabbit afucosyl B antigen (6) Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
I.	Forssman (12) GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-ceramide
J.	Agalactosyl-norhexaosylceramide (12) GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
K.	Le^x pentasaccharide (4) Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
L.	Norhexaosylceramide (18) Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
M.	Dimeric Le^x (9) Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-ceramide
N.	GM_3 (8) NeuAcα2-3Galβ1-4Glcβ1-ceramide
O.	Sialylparagloboside (8) NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide

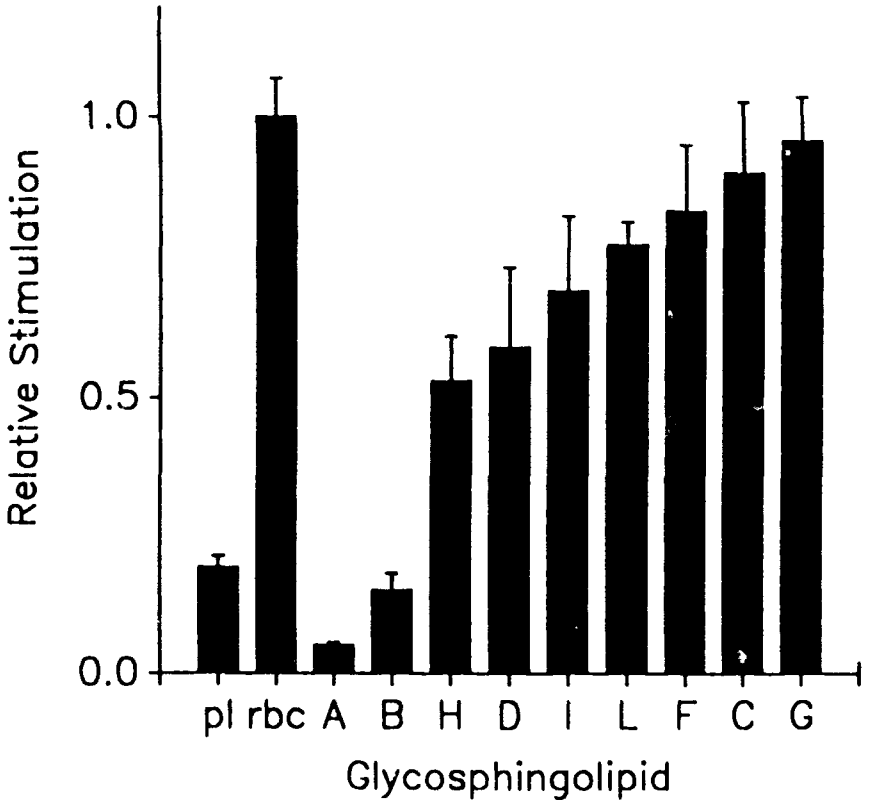

Fig. 1. Enhancement of liposome stimulated *E. histolytica* actin polymerization by Gal and GalNAc terminal straight chain glycosphingolipids. The key to lettered glycosphingolipids is given in Table 1.

enzyme/10^8 beads in 0.1 M citrate, pH 4, for 4 hr at 37° C) followed by washing with phosphate buffered saline, pH 6.3 (PBS). The effectiveness of glycoprotein conjugation and galactose hydrolysis was estimated by fluorescence cytometry of beads after binding of 0.1 μg/ml of the β-galactoside specific lectin, FITC-Allo-A (E-Y Laboratories, Inc., San Mateo, CA) and washing with 0.01% sodium dodecylsulfate to remove non-specifically bound lectin. A fluorescence signal well above that of the unconjugated beads was obtained with all glycoprotein conjugated beads. The integrated signal of the asialofetuin conjugated beads was greatest; this was reduced 17% by treatment with β-galactosidase ($P < 0.001$).

Preparation asialoglycophorin liposomes. Asialoglycophorin (Galβ1-3GlcNAc termini) liposomes were prepared by dialysis of lipid-protein suspensions as follows: Membrane lipids (Sigma,) formulated as described above and containing 0.1 μci ^{14}C-phosphatidylcholine to monitor the total lipid concentration, were dried on the wall of a glass test tube. Human asialoglycophorin A (Sigma; 0.1 mg/mg lipid) was dissolved in 0.1% Triton X-100 (1.8 ml/mg protein, containing 5 mg/ml carboxyfluorescein if required) then 1/10 volume of 10-times concentrated PBS was added. The protein was suspended with the lipids by brief sonication and dialyzed for 48 hr against several changes of PBS containing Biobeads SM-2 (Bio-Rad, Rockville Centre, NY). The liposomes were collected and washed once with PBS by centrifugation at 100,000 X g for 40 min and finally suspended in PBS at a concentration of 4.5 mg total lipid/ml. Asialoglycophorin incorporation into liposomes was determined by protein assays and ranged from 60% - 75% of the calculated maximum in the preparations used. Protein-free liposomes used in this study were prepared by the same procedure.

Results

Stimulation of *E. histolytica* actin polymerization by liposomes. The glycosphingolipids assessed in this study are listed in Table 1 with a key to the letter codes used in the Figures. The number of actin stimulation assays conducted with each glycosphingolipid is indicated in parentheses in Table 1.

The ability of Gal and GalNAc terminal straight chain glycosphingolipids to enhance liposome activated *E. histolytica* actin polymerization is shown in Fig. 1. Stimulation by synthetic liposomes lacking added glycosphingolipid (pl) averaged 19% of

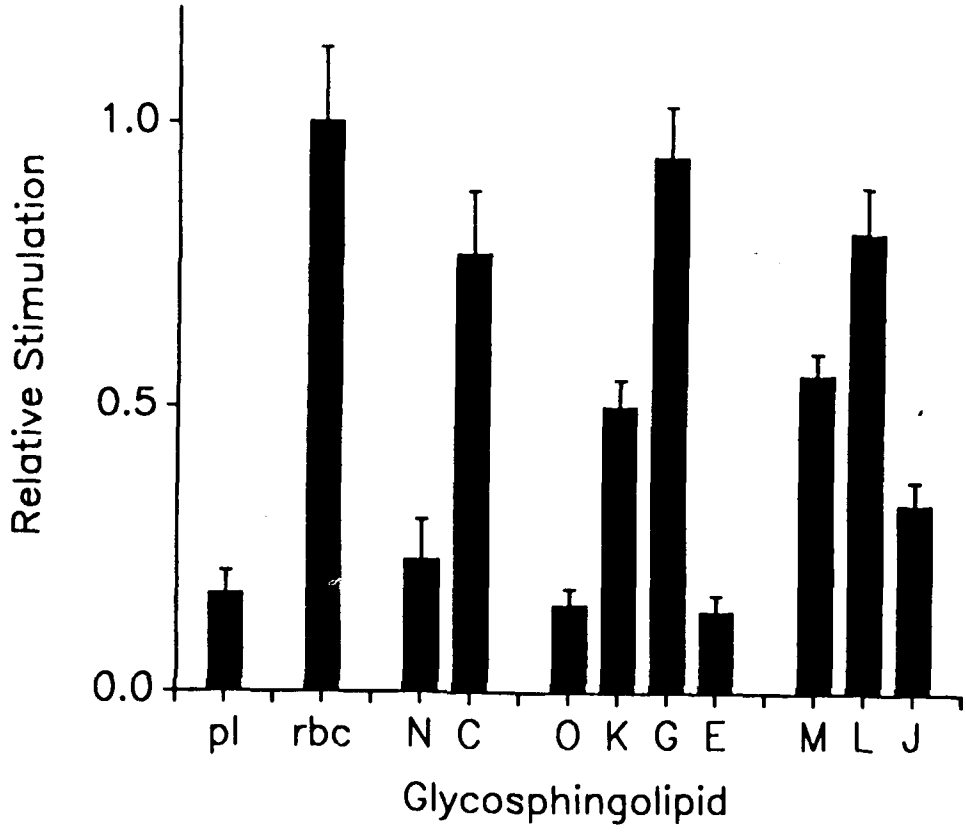

Fig. 2. The effects of structural modifications on the ability of glycosphingolipids to enhance liposome stimulated *E. histolytica* actin polymerization. The key to lettered glycosphingolipids is given in Table 1.

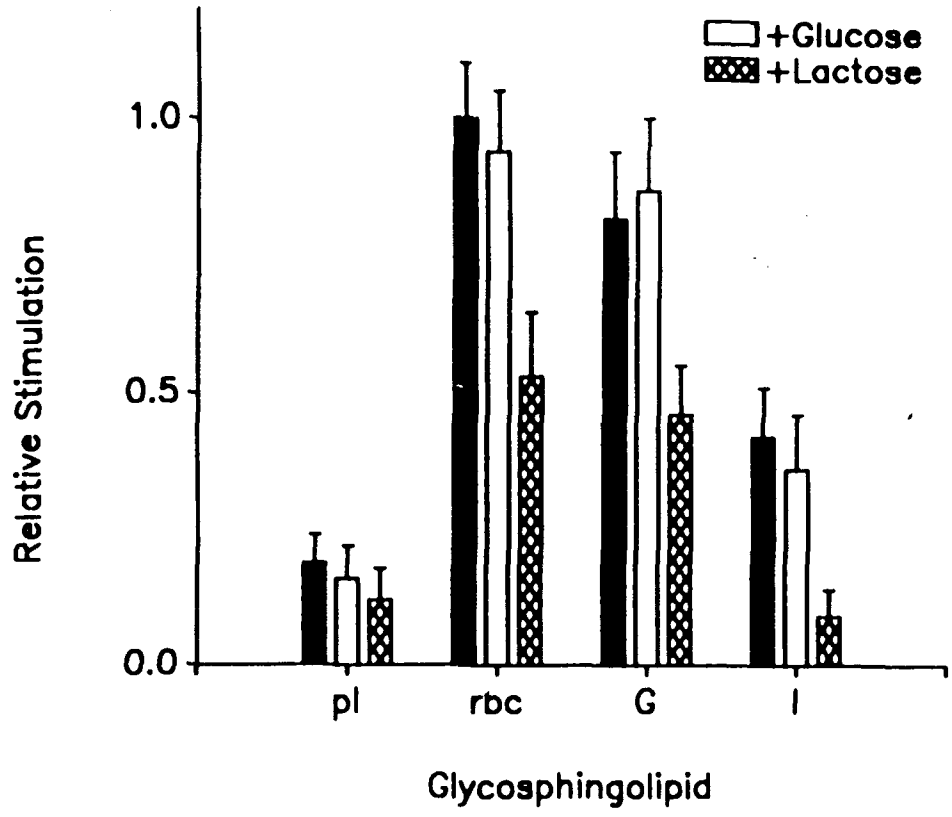

Fig. 3. The effects of glucose (50 mM) and lactose (50 mM) on the ability of RBC membrane (rbc), paragloboside (G) and Forssman (I) bearing liposomes to stimulate *E. histolytica* actin polymerization.

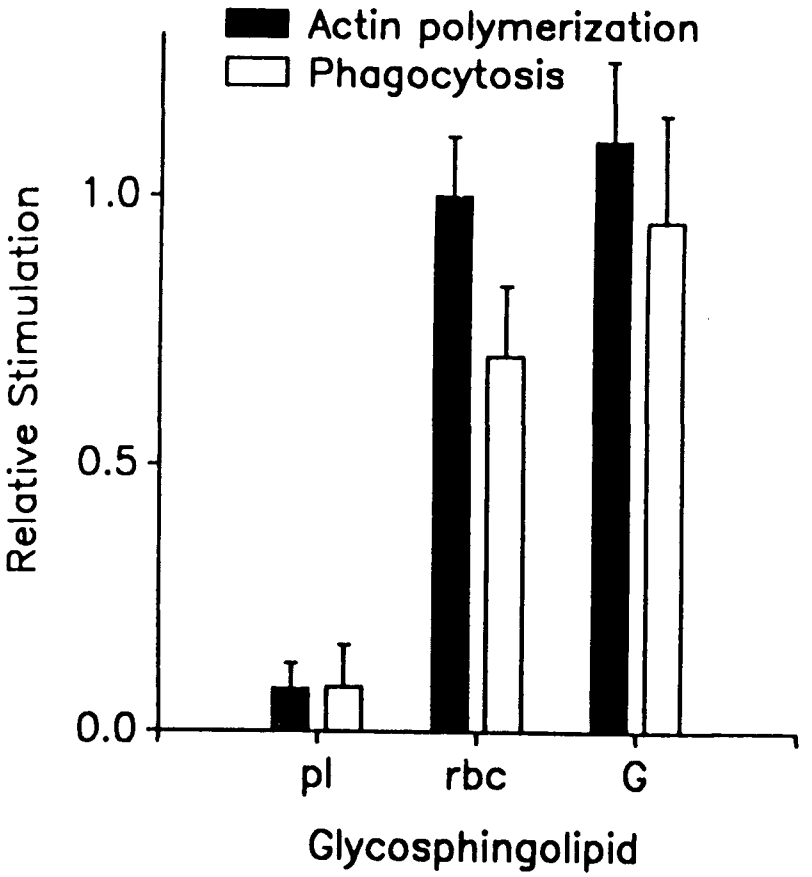

Fig. 4. Correlation of liposome stimulated *E. histolytica* actin polymerization with liposome phagocytosis. G, paragloboside.

the stimulation by liposomes prepared from the total lipid extract of RBC membranes (rbc). Synthetic liposomes constructed with either of the monohexosylceramides, glucosylceramide (A) or galactosylceramide (B), were without stimulatory activity. In fact, glucosylceramide depressed stimulatory activity below that of the glycolipid-free liposomes ($P<0.01$).

All Gal or GalNAc terminal glycosphingolipids bearing two or more sugars enhanced the ability of the synthetic liposomes to stimulate *E. histolytica* actin polymerization compared to the glycolipid-free liposomes ($P < 0.001$). Glycans with a β1-4 or 1-3 terminal glycosidic bond (L, F, C, G) were more effective than glycans with a terminal α1-4 or 1-3 linkage (H, D, I). Liposomes constructed with trihexaosylceramide (D, Galα1-4 terminus) and rabbit afucosyl B antigen (H, Galα1-3 terminus) were only about 50% as effective as RBC membrane liposomes ($P < 0.05$).

The kinetics of synthetic liposome stimulated cytoskeleton activation were determined using paragloboside liposomes. As with RBC membrane liposomes (2), the maximum stimulation was reached between 1 and 2 min following challenge (data not shown). No stimulation of actin polymerization occurred with paragloboside bearing liposomes if phosphatidylserine was omitted from the liposome formulation, verifying the requirement for a negatively charged phospholipid for this response (2).

The effects of modification of glycosphingolipid glycan structure on liposome stimulatory activity are shown in Fig. 2. NeuAc attached to the terminal Gal of lactosylceramide or paragloboside (GM_3 [N] and sialylparagloboside [O], respectively) essentially eliminated the enhancing activity of the these glycosphingolipids. Fucose, attached to the penultimate sugar (GlcNAc) of paragloboside (Le^x pentasaccharide, K) or both GlcNAc residues of norhexaosylceramide (dimeric Le^x, M), was less hindering, but diminished the activity of the corresponding straight chain glycosphingolipids 56% and 38%, respectively ($P < 0.05$).

Finally, removal of the terminal Gal from paragloboside and from norhexaosylceramide, which produced (GlcNAc)trihexaosylceramide (E) and agalactosyl-norhexaosylceramide (J), respectively, eliminated the stimulatory activity of the former and reduced that of the latter 61% ($P < 0.001$). However, agalactosyl norhexaosylceramide still

Table 2. Adherence and phagocytosis by *E. histolytica* of protein conjugated latex beads and erythrocytes.

Glycoprotein conjugate (Glycan Terminal Sequence)	Adherence	Phagocytosis	Adherence + Phagocytosis
None	55.8 ± 3.9	0.0	55.8
Fetuin (NeuAc-Galß1-4GlcNAc-)	82.6 ± 3.2	10.0 ± 2.8	92.6
Asialofetuin (Galß1-4GlcNAc-)	147.8 ± 5.0[a]	15.6 ± 3.0	163.4[c]
Agalactosylasialofetuin (GlcNAc-)	95.8 ± 3.9	8.0 ± 1.9	103.8
Whole RBC	14.4 ± 3.8	268.0 ± 8.3[b]	282.4[b]

* Values are means ± SEM for 5 assays of the number of beads attached and phagocytized per 100 amoebae. Significance was determined using the student's t-test.

[a] Significantly greater than all other bead targets ($P < 0.01$).

[b] Significantly greater than all bead targets ($P < 0.001$).

[c] Significantly greater than the unconjugated and fetuin conjugated beads ($P < 0.01$).

Table 3. Stimulation of *E. histolytica* actin polymerization by various target models.

Target		Relative Stimulation
A.	Asialofetuin beads	0.00
B.	Asialoglycophorin liposomes (0.6 mM PS)	0.74 ± 0.12[a]
C.	Asialoglycophorin liposomes (no PS)	0.00
D.	Glycolipid-free liposomes (0.6 mM PS)	0.26 ± 0.07
E.	Glycolipid-free liposomes (0.12 mM PS)	0.45 ± 0.09[b]
F.	Glycolipid-free liposomes (0.18 mM PS)	0.52 ± 0.08[b]
G.	Paragloboside liposomes (0.6mM PS)	0.90 ± 0.11[a]
H.	Erythrocyte membrane liposomes	1.00 ± 0.16[a]

Values are means ± SEM of the relative fraction of stimulated amoebae in 100 cells counted compared to amoebae challenged with erythrocyte membrane liposomes.

[a] Significantly greater than E ($P < 0.001$).

[b] Significantly greater than E ($P < 0.01$).

enhanced liposome stimulatory activity compared to the glycolipid-free controls ($P < 0.01$).

Sugar inhibition of glycosphingolipid stimulated actin polymerization. Lactose (Galβ1-4Glc), known to exhibit structurally specific inhibition of *E. histolytica* interaction with whole target cells, and glucose, ineffective as an inhibitor, were tested for their ability to inhibit amoeba actin polymerization stimulated by liposomes prepared with lycosphingolipids of opposite terminal glycoside configuration -- paragloboside (Galβ1-4GlcNAc) and Forssman (GalNAcα1-4Gal). The results are shown in Fig. 3. Lactose, but not the control sugar, inhibited the activity of RBC membrane and paragloboside (G) liposomes similarly, near 40%, and blocked the weaker stimulation by Forssman (I) liposomes 70% ($P < 0.01$).

Correlation of stimulation of actin polymerization with phagocytosis. The relative stimulation of actin polymerization reflected the relative extent of vesicle phagocytosis for pl, rbc and paragloboside (G) liposomes (Fig. 4). It was expected that a similar correlation would have been observed for the other synthetic liposome types, but this was not tested.

Recognition and binding of glycoprotein conjugated beads. Recognition specific binding of glycoprotein conjugated beads was demonstrated by the results shown in Table 2. The known target cell carbohydrate specificity of *E. histolytica* predicts that the Galβ1-4GlcNAc terminal sequence of the asialofetuin beads would be the best recognized. Adherence to *E. histolytica* of asialofetuin beads was significantly greater than that to unconjugated, fetuin or agalactosylasialofetuin conjugated beads ($P < 0.01$). This implied that the terminal Gal residues of the asialofetuin conjugated beads were specifically recognized by the amoeba lectin. Failure of the recognized glycoprotein conjugated beads to stimulate *E. histolytica* actin polymerization is shown in Table 3. No increase in amoeba polymerized actin was detected after challenge for 2, 4 and 10 min with asialofetuin conjugated beads (Table 3, A). Thus, lectin binding of recognized carbohydrates even when presented on the surface of particulate target of suitable size, is not sufficient to stimulate the amoeba cytoskeleton response.

Only a small percentage of the attached asialofetuin beads were phagocytized (Table 2). Under identical assay conditions

erythrocyte phagocytosis occurred quickly following contact. If it is assumed that adherence to the Gal specific lectin precedes phagocytosis, then the total number of adhered erythrocytes was approximately twice that of asialofetuin beads, while the number phagocytized was 17-fold greater. Thus, while phagocytosis may be enhanced by lectin binding, it is not a direct consequence of adherence.

The ability of glycoprotein bearing liposomes and liposomes of varying negative phospholipid content to stimulate actin polymerization is shown in Table 3. Liposomes constructed with asialoglycophorin, an erythrocyte integral membrane glycoprotein with Galβ1-3GlcNAc termini, stimulated significant amoeba actin polymerization (Table 3, B) compared to the carbohydrate-free liposomes of the same phospholipid composition (Table 3, D; $P < 0.001$). We have shown previously, that liposomes lacking a negatively charged phospholipid did not stimulate actin polymerization, even when they contained a recognized glycosphingolipid (3). Likewise, asialoglycophorin liposomes lacking a negatively charged phospholipid did not stimulate actin polymerization (Table 3, C). They did, however, bind to the galactose lectin on the amoeba surface. This was demonstrated using carboxyfluorescein loaded liposomes. Under the fluorescent microscope carboxyfluorescein loaded asialoglycophorin liposomes lacking phosphatidylserine, but not similar glycoprotein-free liposomes, were clearly seen to bind to the amoeba surface when added under the coverslip of a fresh mount of *E. histolytica* trophozoites washed and suspended in PBS. This attachment was completely eliminated by prior addition to the amoeba suspension of 50 mM lactose, a recognition specific inhibitor (3), but not by addition of the same concentration of the nonspecific sugar, glucose. Therefore, the failure of the uncharged asialoglycophorin liposomes to stimulate amoeba actin polymerization was not due to a failure to bind to the amoeba lectin.

Increasing the negatively charged phospholipid concentration of liposomes partially obscured the enhancing effect of incorporated Gal terminal glycans on amoeba actin polymerization. Carbohydrate-free liposomes containing 8.6 mol % phosphatidylserine (Table 3, D) had about one-fourth the stimulating activity of the same liposomes containing, in addition, 10 mol % paragloboside (Table 3, G). The activity of the glycan-free liposomes was increased by increasing the concentration of negatively charged phospholipid (Table 3, E, F).

Discussion and conclusions

We showed previously that liposomes prepared from RBC membrane lipids mimicked whole RBCs in their ability to elicit rapid contact dependent actin polymerization and phagocytosis by *E. histolytica* (2). In the present study we have demonstrated that the same cellular responses are triggered by a variety of synthetic liposomes containing glycosphingolipid glycans. The similarities in the responses to synthetic and cell membrane liposomes included the kinetics and extent of cytoskeleton activation, a correlation of the cytoskeleton response with target phagocytosis and inhibition by a disaccharide (lactose) that blocks interaction of the parasite with cell membrane liposomes and whole cells. Based on this evidence, we have concluded that the relative degree to which synthetic liposomes stimulated amoeba actin polymerization reflected the relative specificity of the parasite for interaction with the lipid associated glycoconjugates expressed on these vesicles.

E. histolytica recognized a variety of glycosphingolipid glycans with Gal or GalNAc terminal residues. A β1-4 or 1-3 terminal glycosidic bond was favored. Glycosphingolipids with an α1-4 or 1-3 terminal linkage possessed some, but weaker, stimulatory activity. The structure of the penultimate sugar of the glycoconjugate was not critical for recognition, since this position was occupied by GlcNAc, Glc, GalNAc and Gal in different stimulatory glycans. The failure of galactosylceramide to enhance liposome stimulated actin polymerization implies that a disaccharide glycan is minimally essential. However, as suggested earlier (2), the failure of this monohexosylceramide to stimulate a response may have been due to hindrance of carbohydrate binding interactions close to the lipid bilayer. The apparent inhibition of the basal activity of glycolipid-free liposomes by glucosylceramide is intriguing, but unexplained.

Terminal GlcNAc residues appeared not to be recognized. Removal of the terminal Gal from paragloboside eliminated the activity of this, the most stimulatory of the glycosphingolipids tested. Loss of the terminal Gal from norhexaosylceramide reduced its activity by 61%. The residual activity of agalactosyl-norhexaosylceramide may have reflected recognition of the internal Galβ1-4 residue of that glycan.

NeuAc attached to the Gal terminus of an otherwise stimulatory glycosphingolipid or fucose attached to the next proximal sugar significantly reduced stimulatory activity. With fucose, this was probably due primarily to steric hindrance of binding to the terminal sugar; in the case of the negatively charged NeuAc, repulsive charge effects may be involved, since the liposomes also carry a net negative charge.

Our results are generally consistent with those obtained in the studies of *E. histolytica* adherence to CHO cell surface glycosylation mutants (9, 10, 14). Interaction was always greatest with Galβ1-4 terminal glycans unencumbered by attached NeuAc or fucose residues. Interaction with GlcNAc terminal glycans was low. In addition we have detected recognition of terminal Gal(GalNAc)β1-3 and Gal(GalNAc)α1-4 or 1-3 glycans.

Li *et al.* (9) proposed that terminal Galβ1-4GlcNAc units represented the principal carbohydrate structure recognized by *E. histolytica*. This conclusion was based on the observation of maximal adherence of amoebae to mutants with increased Galβ1-4GlcNAc termini and, of a number of sugars tested, the strongest inhibition of adherence (9) and cytolysis (10) by LacNAc. Ravdin and co-workers reached the same conclusion (14). Our results support this, but also have demonstrated by analysis of individual glycans that other terminal sequences and configurations are recognized, at least when presented as glycosphingolipids in the liposome model.

While other interpretations are possible, the ability of lactose to inhibit amoeba interaction with both paragloboside and Forssman glycosphingolipid bearing liposomes implies that a single binding protein was responsible for recognition of all the glycosphingolipids we tested. A logical candidate is the Gal/GalNAc binding protein which has been studied extensively and isolated by Petri and co-workers (12). The greater percentage inhibition and weaker cytoskeleton stimulating activity of glycans with an α-linked terminal glycoside imply weaker affinity of the binding protein for this configuration than for β-linked terminal residues. A further implication of our results, in general, is that binding occurs primarily to the terminal sugar(s) of lipid glycans.

Previous investigations have focused on recognition of mammalian cell glycoproteins by *E. histolytica*. Recognition specific binding and ingestion of galactose terminal intestinal mucins has

been demonstrated (), and we have shown enhanced attachment and phagocytosis by *E. histolytica* of latex beads conjugated with galactose terminal glycoproteins (Bailey, G. B., *et al.*, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989). The present study demonstrates that glycosphingolipids prepared from target cell membranes are recognized by the parasite. Because of common steps in biosynthesis, some mutations cause the same alteration of both protein and lipid glycoconjugates in CHO cells (16). When such mutants have been used in studies of *E. histolytica* carbohydrate recognition specificity, the results may have reflected alterations in glycosphingolipid as well as glycoprotein glycan structure.

Because glycolipid-free liposomes stimulate some amoeba actin polymerization, and because stimulatory glycosphingolipid bearing liposomes lacking a negatively charged phospholipid are inactive, it is clear that binding of glycosphingolipid glycans *per se* to amoeba proteins does not trigger the parasite cytoskeleton response. Presumably, glycan binding facilitates interactions with other molecules of the vesicle lipid bilayer. It is important now to determine whether *E. histolytica* distinguishes glycoprotein from glycosphingolipid glycans on the surface of mammalian cells and, if so, the relevance this has to the mechanism of target cell attack by the parasite.

We interpret these results as follows: Target cell surface glycan binding to the *E. histolytica* galactose lectin is responsible for attachment of target cells but is not directly involved in the mechanism of amoeba cytoskeleton activation. Both glycoprotein and glycolipid glycans participate in target binding. The transmembrane signals that lead to *E. histolytica* actin polymerization following target contact are triggered, at least in part, by negatively charged lipids of the target plasma membrane. Glycan binding enhances the efficiency of interaction with lipids, thence, the degree of cytoskeleton response, possibly by facilitating closer or longer association of target lipids with reacting sites on the amoeba surface. Increasing the concentration of negatively charged lipid in the glycan-free liposomes mimicked the effect of glycans simply by increasing the percent saturation of lipid reacting sites on the amoeba surface. Further elucidation of the role of target cell membrane lipids in the mechanism of *E. histolytica* cytoskeleton activation should provide useful information about the molecular mechanism of *E. histolytica* cytopathogenicity.

Section 2.

This section describes the continuing studies of the ability of drug loaded liposomes to selectively destroy *E. histolytica* .

Introduction.

Contact with mammalian target cells (e.g., erythrocytes) *in vitro* stimulates rapid polymerization of *E. histolytica* actin and phagocytosis of the target. This same response is triggered by liposomes prepared from erythrocyte membranes or synthetic liposomes containing galactose terminal glycolipid or glycoprotein glycans (which bind to a galactose specific amoeba lectin) and negatively charged phospholipid (which stimulates cytoskeleton activation and phagocytosis).

Liposomes have been employed successfully as drug carriers for the treatment of infections by intracellular pathogens. In these instances the drug is targeted to the infected cells by the propensity of the latter to interact with or ingest the lipid vesicles. Thus, the drug is concentrated within the infected cells and, thereby, brought to bear upon the invading pathogen. It occurred to us that the specific recognition and phagocytosis by *E. histolytica* of appropriately constructed liposomes might be exploited similarly, but, in this case, to destroy the ingesting cell itself.

Metronidazole is generally accepted as the drug of choice for the treatment of invasive amebiasis. However, due to its rapid absorption, it is least effective against parasites resident in the intestinal lumen. For this reason, poorly absorbed, albeit less potent amebicides, are often employed with, or in place of metronidazole to treat intestinal infections. Pathogenic amoebae remain resident in the intestine of the majority of amebic patients even following successful treatment of clinical symptoms; these are often the cause of reinvasion of the host and spread of the disease. Thus, while existing amoebic drug therapies are effective for clinical cure, there is reason to seek more efficient means of drug delivery, particularly to treat intestinal infections and to rid the patient of remaining parasites.

In this initial study we have compared the ability of metronidazole loaded, recognition specific liposomes and comparable free metronidazole solutions to inactivate *E. histolytica*

trophozoites *in vitro* and to protect mammalian cell cultures from destruction by the parasite. The results suggest that this means of drug delivery should be further explored as a possible strategy for the treatment of amebiasis.

Body

Experimental methods.

Cells. *E. histolytica*, strain HM1-IMSS, was cultured axenically in TYI-S-33 medium as described. Chinese hamster ovary (CHO) cells (ATCC CCL 61) were grown with F-12 medium to confluency in 96-well plates.

Preparation of drug loaded liposomes. Liposomes were prepared by sonication of dried human type A erythrocyte membrane lipid extracts in PBS as previously described. Metronidazole, and ^{14}C-glucose (10 μci/ml) when required, were dissolved in the PBS prior to sonication to load liposomes. Unencapsulated components were removed by sedimentation of the liposomes at 40,000 X g for 30 min through a 5-fold excess of PBS. The liposome pellet was suspended in PBS at 4.5 mg total lipid/ml and stored at 5^{o}C until used.

Assay of amoeba inactivation by metronidazole. *E. histolytica* trophozoites in mid-growth phase were harvested by centrifugation at 250 X g for 5 min, washed and then suspended at 2 X 10^4 cells/ml in TYI (culture medium minus serum). Two ml of amoeba cell suspension were placed in 2.5 ml screw capped vials to which 1/10 volume of drug loaded liposomes, drug free liposomes, metronidazole dissolved in TYI or TYI alone was then added. The vials were incubated horizontally at 37^{o}C with gentle resuspension of cells at 15 min intervals. Loss of trophozoite activity was quantitated directly in vials using an inverted microscope; spherical, nonmotile cells were scored as inactive. Cell viability was determined by carboxyfluorescein retention after incubation of samples of the above cell suspensions for 10 min with 10 μg/ml diacetylcarboxyfluorescein. In both assays one hundred cells in randomly selected fields were counted for each data point.

Assay of CHO cell release. The ability of *E. histolytica* trophozoites to release attached CHO cells from the surface of wells of 96-well plates provided a means to measure the destruction of this mammalian target cell model. Culture medium

was decanted from the CHO cell monolayers and replaced with 200 μl of amoeba cell suspension that had been pre-exposed for 1 hr to the desired drug or control treatment in vials as above. The plates were covered and the amoebae were immediately sedimented upon the monolayer by centrifugation at 250 X g for 1 min. The plates were incubated for 2 hr at 37°C. Amoebae and released CHO cells were then removed by three washes with ice cold PBS.

The remaining attached CHO cells were incubated for 45 min at 37°C with 200 μl Hoescht 33342 (Sigma, 10 μg/ml in serum free F-12 medium) to stain DNA. Bound fluorescence was quantitated using a Dynatech 96-well plate fluorescence reader. Percent CHO cell survival was expressed as 100 X (test well fluorescence - background fluorescence) ÷ (control well fluorescence - background fluorescence), where the control well contained CHO cells treated with TYI and the background fluorescence was the reading for a well containing Hoescht dye but no cells. Each value in a single experiment was the mean of readings from 10 replicate wells. For statistical calculations these means were considered as a single experimental data point. Means and error bars in figures are given ± SEM. Statistical significance was determined using the Student's t-test.

Results.

Drug loading of liposomes. By quantitation of ^{14}C-glucose co-encapsulated with metronidazole, we determined that the efficiency of liposome loading was 2.5 ± 0.4% using 4.5 mg erythrocyte membrane lipid/ml. Less than 5% of the encapsulated radioactivity was released from vesicles stored for 24 hr at 5°C. Nevertheless, liposome suspensions were fleshly prepared for each experiment. The equivalent free drug solution concentration in assays (the free solution concentration that would obtain if all the encapsulated metronidazole were released) was calculated as 0.025 X the concentration of metronidazole used to load liposomes/the fold dilution of the liposome suspension in the assay. Metronidazole was soluble in PBS to 4 mg drug/ml. Therefore, this was the highest drug concentration used to load liposomes and yield an equivalent free drug solution concentration of 10 μg/ml after 10-fold dilution of liposomes.

Drug inactivation of *E. histolytica* trophozoites. At an equivalent free drug solution concentration of 10 μg metronidazole/ml, both drug loaded liposomes and free metronidazole solutions inactivated *E. histolytica* trophozoites

essentially 100% within 3 hr (Fig.1). The rate of inactivation was somewhat faster with soluble metronidazole. With longer incubation times the rounded cells became distended, endoplasm separated from the plasma membrane and frank cell lysis became increasingly evident.

After 2 hr in the presence of drug more than 80% of the amoebae, including those inactivated, were viable as assessed by carboxyflourescein retention; after 3 hr more than 50% still retained the dye. With longer exposure to drug increasing numbers of cells became nonvialble. Drug free liposomes had no effect on amoeba activity or survival over the same time period.

Based on these results 1 hr preincubation of amoebae with drug was selected for the CHO cell protection assays. At this time, 75% of the amoebae incubated with free metronidazole and over 90% of those incubated with drug loaded liposomes were still active; nearly all cells in both treatments were viable as assessed by carboxyfluorescein dye retention. The CHO cells were challenged with amoebae for 2 hr. Therefore, the amoebae had the opportunity to act upon the mammalian cell monolayer during the period of most parasite inactivation, but while they were still alive.

Drug protection of CHO cell release.

The amount of drug delivered to *E. histolytica* trophozoites in liposomes was varied in two ways in the CHO cell protection experiments; trophozoites were exposed either to a constant concentration of liposomes loaded with different concentrations of metronidazole, or to different concentrations of liposomes loaded with the same concentration of metronidazole. In the former case the total erythrocyte membrane lipid concentration in assays was 0.45 μg/ml, which we have shown elicits a maximum *in vitro* phagocytic response by *E. histolytica*.

The ability of amoebae to destroy CHO cell monolayers after exposure to liposomes loaded with a range of metronidazole concentrations and to the equivalent free drug concentrations of metronidazole is shown in Fig. 2. Means of 93% and of 89% of the mammalian cells were released from the substratum by untreated amoebae and amoebae exposed to drug free liposomes, respectively. This difference was not statistically significant. Amoebae exposed to 2.5 μg or more metronidazole/ml, whether the drug was encapsulated or in solution, destroyed significantly fewer CHO

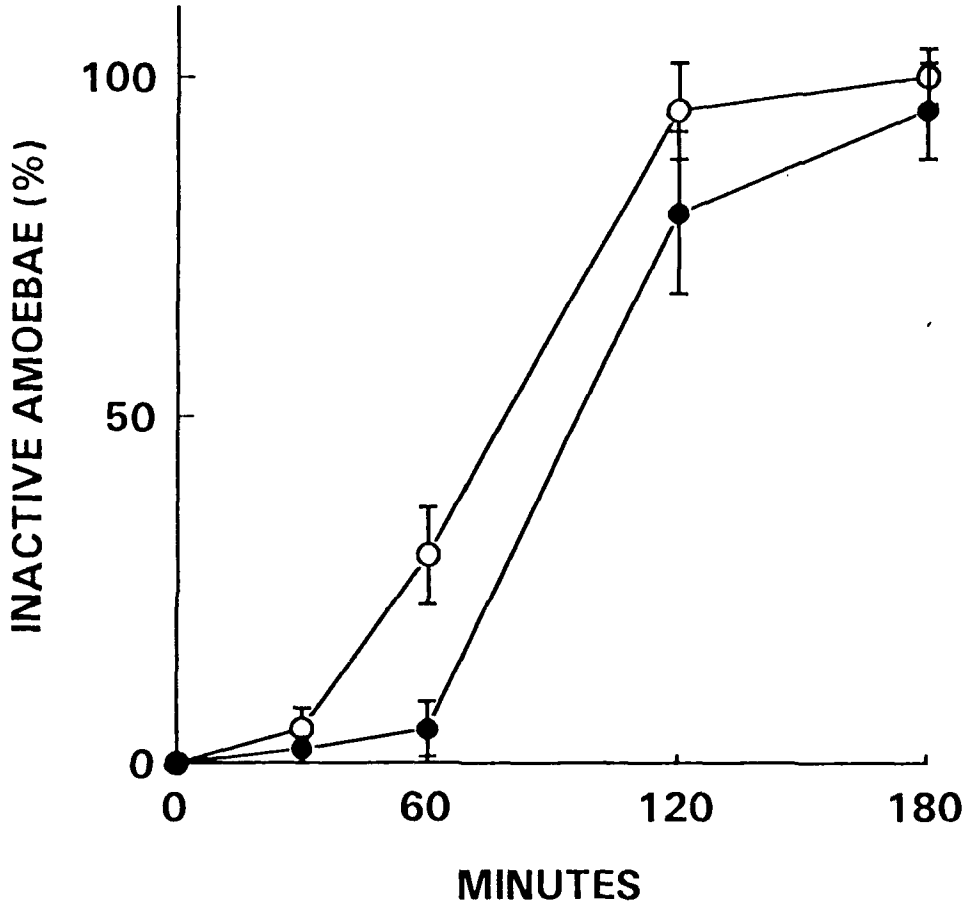

Figure 1. Time course of destruction of *E. histolytica* trophozoites by metronidazole loaded liposomes (open circles) and equivalent free metronidazole solutions (closed circles).

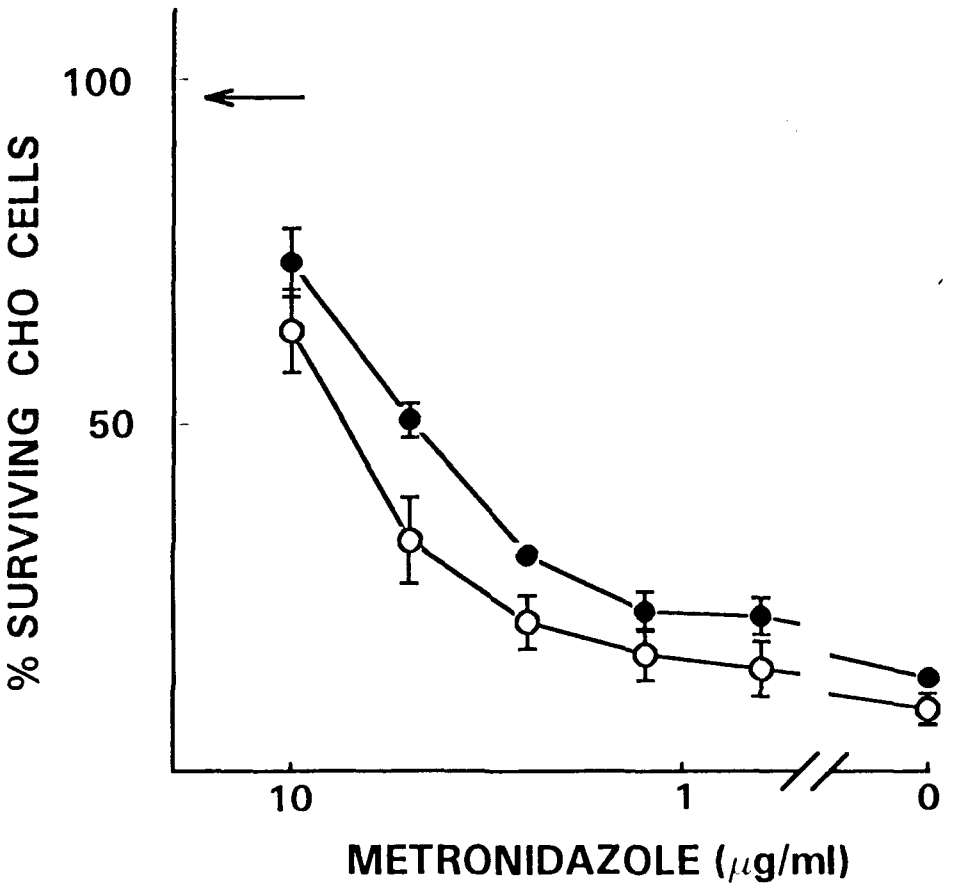

Figure 2. Ability of metronidazole loaded liposomes (open circles) and equivalent free metronidazole solutions (closed circles) to protect CHO cells against destruction by *E. histolytica* trophozoites. Metronidazole concentrations in liposomes were varied by change the concentration of metronidazole loaded into liposomes prepared at a constant lipid concentration (4.5μg/ml).

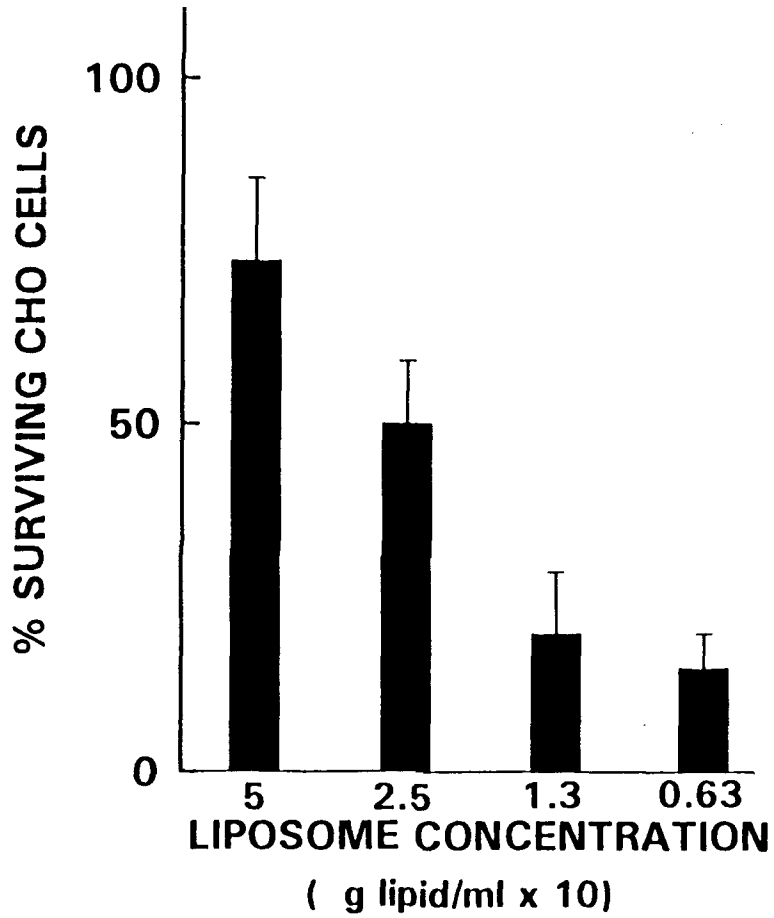

Figure 3. Ability of metronidazole loaded liposomes to protect CHO cells against destruction by *E. histolytica* trophozoites. Metronidazole concentrations were established by varying the amount of liposomes loaded with a constant concentration of metronidazole (4mg/ml)

cells in that time ($P < 0.05$ for all drug levels). Drug loaded vesicles were modestly more effective than free drug, particularly at the higher drug levels ($P < 0.005$ at 5 μg/ml; $P < 0.01$ at 10 μg/ml).

The destruction of CHO cell monolayers by amoebae exposed to decreasing numbers of liposomes loaded with a solution of 4 mg metronidazole/ml is shown in Fig. 3. Decreasing the liposome concentration one-half significantly reduced their protective ability. However, protection remained significantly greater than the drug free controls down to one-fourth the liposome concentration that elicits the maximum phagocytic response ($P < .05$). At the reduced liposome concentrations the protective effect remained comparable to that of the same equivalent free drug solution concentrations (data of Fig. 2).

Discussion and Conclusions.

Metronidazole loaded liposomes constructed from erythrocyte membrane lipids were as effective as solutions containing the same total content of drug in inactivating *E. histolytica* trophozoites *in vitro*. The protective efficacy of the drug loaded liposomes was demonstrated by their ability to block parasite destruction of mammalian cell monolayers. Again this was comparable to the efficacy of equivalent free drug solutions. At a liposome concentration that saturates the *in vitro* phagocytic capacity of the amoebae, the drug loaded vesicles provided slightly greater protection than equivalent strength free drug solutions, at least at higher dose levels.

The rate of release of radioactive glucose from liposomes demonstrated that spontaneous leakage or release of liposome contents into the extracellular environment during interaction with amoebae was not significant, at least over the time period of the experiments. Therefore, extracellular release of metronidazole and subsequent uptake of drug from free solution would appear not to account for the fact that the efficacy of liposome drug delivery was about the same as that of the comparable free drug solutions. More likely, ingestion and intracellular release of drug occurred. This is consistent with our earlier observations that phagocytosis of recognized vesicles occurs rapidly following contact with trophozoites, and that internalized liposomes retain encapsulated substances. The mechanism of phagosome processing within *E. histolytica* is not

understood. However, it is clear that access of drug to the site(s) of action was sufficient to effect inactivation with the same kinetics as a drug absorbed from free solution.

Most studies of the effect of drugs on *E. histolytica* survival have been conducted in growth cultures over a period of one or more days to allow assessment of cell multiplication. We employed a rapid assay procedure in these experiments for several reasons. First, the phagocytic response of *E. histolytica* to whole target cells or recognition specific liposomes is rapid. The course of ingestion is complete within 1 hr following challenge with the target. While continued slow ingestion of liposomes would be expected, maximum uptake of encapsulated drug would be expected over this initial period. At the levels of drug and liposome load used in this study, a significant number of amoebae were inactivated during this time.

Periods of 1 to 6 hr have been used in assays of *E. histolytica* destruction of mammalian cell monolayers. We adjusted the parasite:target cell ratio so that essentially total release of the monolayer occurred within 2 hr in control assays. This allowed assessment of the protective ability of the encapsulated and free drugs during the period of their continuing action upon the parasites. While the effective drug loads were similar to those observed by others *in vitro*, different comparative potencies may have been observed with longer pretreatment periods with drugs at lower doses. Nevertheless, we feel that these preliminary results are sufficiently encouraging to justify more detailed exploration of the feasibility of liposomes as a drug delivery strategy for the treatment of amebic infections in humans.

Refinement of several methodological parameters not addressed in this study might enhance considerably the efficiency of liposome mediated drug delivery and, thereby, their efficacy compared to free drug administration. For example, no attempt was made to seek a solvent system that would maximize solubility of metronidazole while still supporting preparation of stable liposomes. Increasing the encapsulated load would allow delivery of larger doses of drug without increasing the total lipid concentration. Also, drug combinations, such as those employed in the treatment of luminal infections, were not tested. These, particularly if acting upon the amoebae by different mechanisms, could enhance the destructive capacity of individual vesicles.

Liposomes were prepared from erythrocyte membrane lipids by sonication, as in our previous studies. These vesicles are multilaminar and of varied size and are not the most effective delivery vehicle. Furthermore, a liposome formulation for administration to humans would have to be more explicitly defined as well as non-immunogenic. We have shown that synthetic liposomes bearing a β-galactose terminal glycolipid glycan and bearing negatively charged phospholipid are as effective as target cell membrane liposomes in stimulating phagocytosis (reported in Section 1). Thus, it is possible to construct chemically defined synthetic liposomes of optimal composition for recognition and ingestion. Such liposomes, prepared by more refined methods (e.g., that generate large volume unilaminar vesicles) will provide maximum drug at minimum total lipid concentration. Finally, other factors should be explored, such as the effect of liposome membrane "fluidity" on the efficiency of vesicle uptake and intracellular release of drug. Optimization of all of these factors could lead to a liposome delivery system that exceeded the efficiency of free solutions of comparable total drug load.

Another logical next step in this study is to assess the efficacy of encapsulated drugs for the elimination of *E. histolytica* infections from experimental animals. Since current drug amebiasis drug therapies are least effective against intestinal infections, it would seem most advantageous if liposome mediated therapy could be developed to treat luminal infestations. Here the questions of liposome dose, mode of administration, stability *in situ* and accessibility to the parasite must be addressed. These investigations are underway.

Note: Since this section was completed we have determined that metronidazole is lost rapidly from liposomes, even though the ^{14}C-glucose tracer was totally retained. We have obtained metronidazole-phosphate, a highly water soluble drug derivative, as a gift from the Upjohn Company. Initial tests indicate that this drug is retained by liposomes. This is being confirmed in the continuing studies. The parasite killing and cultured cell protection experiments will then be repeated with metronidazole-phosphate. The above text is the draft of a manuscript prepared before it was determined that metronidazole was lost from liposomes. This explains why the drug loaded liposomes had the same efficacy as the equivalent free drug solutions. The manuscript will be revised based on the data obtained with metronidazole-phosphate and submitted for publication.

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Section 3. This section describes efforts to develop a suitable animal model for intestinal amebiasis.

Introduction/Scope/Conclusions.

A number of animal models have been used in attempts to establish experimental amebiasis models that could be used to evaluate the in vivo effectiveness of various liposome-administered antiparasitic agents. Three species were employed: the rat, the hamster and the gerbil. In each of the species the animal was first lightly anesthetized with intraperitoneal pentobarbital, a midline incision was made, and axenically cultivated *E. histolytica* trophozoites in TYI-S medium were inoculated directly into the cecum. A previous study (Leitch, 1988) suggested that trophozoite survival would be longest in the gerbil cecum, while one report in the literature (Anaya -Velasquez et al., 1985) suggested that amebic mucosal ulcers might be expected in the hamster cecum. The scoring method of Neal (1951) was used to determine if trophozoite infection affected either the constituency of the luminal content or the thickness of the mucosa. At various intervals after the trophozoite inoculation cecal and colonic contents were taken and examined for viable trophozoites; mucosal tissue samples were taken and prepared for histological and scanning electron microscopic survey of amebic lesions. The cecal content was less viscous in infected animals when compared to uninoculated control animals, but not when compared to medium-inoculated animals, suggesting that changes in large bowel flora subsequent to the inoculation of a relatively large volume of medium was

responsible for any observed change in luminal content consistency. This conclusion was further supported by the fact that 1 week after the inoculation of either 106 or 107 trophozoites we were unable to find evidence of *E. histolytica* trophozoites in any of the models used. Similarly, we were unable to find any histological or ultrastructural evidence of Entamoeba-induced mucosal damage in any of the animal models surveyed. It has been claimed that reducing the cecal flora with a poorly absorbed, broad spectum antibiotic will allow both colonization and invasion of the mucosa by axenically cultivated trophozoites. However, in our hands, the use of neomycin at the dose used by Anaya-Velasquez et al. (1985) did not result in either trophozoite colonization or invasion of the hamster cecum.

Neal and others have reported success in establishing intestinal amebiasis in rodent large bowel using xenic cultures of *E. histolytica*, usually inoculated in Robinson's medium. In these preparations the bacteria are some modification of the patient's flora, and may or may not play some key role in either the trophozoite colonization or the invasion. On the assumption that inability to adapt to the bacterial flora was one reason that we were unable to establish infections using the axenically cultured trophozoites, we developed monoxenic cultures of *E. histolytica* using *E. coli*. These monoxenic cultures were then inoculated into hamster and gerbil cecum preparations. As with the axenic preparations, the monoxenic cultures were ineffective at establishing cecal colonization or invasion.

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Graduate Degrees Resulting from this Research

None